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Research Article

Weak anion and cation exchange mixed-bed microcolumn for protein separation

To separate proteins with a wide distribution of p*I*s under the conditions compatible to online tryptic digestion (with preferable pH = 8.0), weak anion and cation exchange chromatography (WAX/WCX) mixed-bed microcolumn has been developed. With a mixture of five proteins with p*I*s ranging from 4.2 to 11.4, the effect of WAX/WCX ratio on the separation performance was investigated, and an optimum packing ratio of 1:1 w/w was obtained. Moreover, the undesirable hydrophobic interaction between the proteins and the stationary phase was suppressed with 10% ACN v/v added in the mobile phases. Under the optimized conditions compatible to tryptic digestion, basic and acidic proteins were resolved simultaneously, with RSDs of relative retention time on six columns less than 6%, indicating the good resolution and packing reproducibility. Furthermore, one RPLC fraction of proteins extracted from rat middle brain and the whole protein mixture extracted from rat liver were analyzed, respectively. The results demonstrated better separation performance on WAX/WCX microcolumns than that on both weak anion exchange chromatography and weak cation exchange chromatography at pH ~8. We anticipate that WAX/WCX microcolumns are promising for the integration of protein separation and tryptic digestion aiming at high-throughput proteome study.

Keywords: Ion-exchange chromatography / Protein separation / Tryptic digestion / Weak anion and cation exchange mixed-bed
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1 Introduction

As a newly emerging and powerful tool, shotgun strategy has been widely adopted for proteome analysis [1]. However, even with multidimensional HPLC separation, it is still challenging to identify hundreds of thousands of peptides in the digest of a complex sample. To achieve comprehensive identification, protein fractionation prior to digestion is of increasing prevalence [2–4]. By fractionation, the sample complexity could be obviously reduced, and the low-abundance proteins with significant biological functions may thus be identified.

Among various fractionation techniques, IEC is a favorable one, due to its high capacity and high resolution [5]. Proteins retained on IEC column can be eluted by an ionic strength gradient, a pH gradient [6] or the combination of both [7, 8]. As a promising alternative, the mixed-bed ion-exchange resin, since the first introduction by Rassi and Horváth [9, 10], has been paid much attention. Recently, Ottens *et al.* established a multi-dimensional platform involving cation and anion exchange chromatography and polyacrylamide gel electrophoresis for differential proteomic analysis [11]. Besides, Motoyama *et al.* developed an anion- and cation-exchange mixed-bed for the separation of peptides and phosphopeptides [12]. Improved peptide recovery over strong cation exchange chromatography (SCX) resins and better orthogonality to RP separation were achieved. Very recently, an online platform including protein fractionation using mixed-bed IEC microcolumns, tryptic digestion followed by HPLC-MS/MS analysis, was constructed in our laboratory for large-scale proteome profiling [13]. The results showed that the mixed-bed IEC column could be directly coupled with trypsin-based immobilized enzyme reactors, which displayed excellent enzymatic activity under neutral pH buffer (pH ~8.0) [14]. However, important factors that affect protein separation on the mixed-bed IEC columns, such as the mobile-phase composition and the packing material ratio, have not been systematically studied.

In this research, detailed investigation of the weak anion and cation exchange chromatography (WAX/WCX) mixed-

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Abbreviations: **SCX,** strong cation exchange chromatography; **WAX,** weak anion exchange chromatography; **WAX/WCX,** weak anion and cation exchange chromatography; **WCX,** weak cation exchange chromatography bed columns was performed. With the optimized packing material ratio and mobile phases compatible to tryptic digestion, both acidic and basic proteins were simultaneously resolved. Moreover, the applicability of mixed-bed columns was demonstrated by the separation of one RPLC fraction of protein sample extracted from rat middle brain and all the proteins extracted from rat liver, suggesting its superiority for complex sample separation under conditions compatible with tryptic digestion.

2 Materials and methods

2.1 Reagents and materials

Myoglobin (horse), cytochrome *c* (bovine heart), lysozyme (chicken egg white) and α-casein (milk) were bought from Sigma (St. Louis, MO, USA). BSA was purchased from Sino-American Biotech (Beijing, China). The WAX-NP5 and WCX-NP5 resin (nonporous, 5 μm) were from Sepax Technologies (Newark, NJ, USA). Protease Inhibitor Cocktail Set 1 and HPLC-grade ACN were obtained from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q system (Millipore, Molsheim, France). All inorganic reagents used were of analytical-reagent grade or higher purity.

2.2 Instrumentation

HPLC experiments were performed on an HPLC system equipped with a dual P230 pump (Dalian Elite Analytical Instruments, Dalian, China), a 4-Line Degaser DG-2080-54 (Jasco, Tokyo, Japan) and a UV Detector K-2501 (Knauer, Berlin, Germany). Echrom 2000 Workstation (Dalian Elite Analytical Instruments) was used for system control and data processing. Eluent splitting prior to sample injection was carried out with a tee union with a fused silica capillary (75 μm id × 1 m, Ruifeng Chromatographic Device, Handan, China).

Peptide separation and identification were performed on microcolumn RPLC (300 id × 150 mm, C18, 5 μm, 200 Å) coupled with Finnigan LCQ^{DUO} quadrupole ion trap mass spectrometer (LCQ-IT MS, Thermo Fisher, San Jose, CA, USA).

2.3 Preparation of mixed-bed microcolumns

A Peeksil tubing (0.3 mm id × 100 mm) was packed with a mixture of weak anion exchange chromatograpy (WAX) and weak cation exchange chromatography (WCX) resins using slurry method. The resins were first dispersed in 500 mM NaCl, followed by ultrasonication for 1 h. Then the slurry was packed at 4500 psi for over 10 h. Finally, the column was flushed by 1 M NaCl before use.

2.4 Preparation of protein samples

Proteins from rat middle brain and liver were extracted with similar protocols. The tissue was first sliced into small pieces and then washed with PBS several times to remove blood. Then, the tissue pieces were suspended in Tris-HCl buffer (pH 7.4) containing 8 M urea and 1% v/v Protease Inhibitor Cocktail Set 1. Followed by homogenization on ice and ultrasonication, the homogenate was centrifuged at 16 000 × *g* for 1 h. The protein concentration in the supernatant was measured with a Bradford assay kit using BSA as a standard.

2.5 HPLC separation conditions

A five-protein mixture was used to evaluate the separation performance of the mixed-bed microcolumns with binary gradient. The mobile phase A was composed of 10 mM Tris-HCl buffer (pH 8.3, compatible to tryptic digestion) with 10% ACN. Mobile phase B was composed of 2 M NaCl in A. Gradient elution conditions were set as follows: 0% B for the first 5 min, and increasing 1% B/min for the next 75 min. The flow rate after splitting was 5 μL/min. Samples were introduced through an injection valve equipped with a 5 μL loop. All percentages presented volume ratio unless otherwise noted.

One hundred microliter of proteins extracted from rat middle brain (~1.5 mg/mL) was separated by a Hypersil C8 column (4.6 mm id × 250 mm, 5 μm, 300 Å). For gradient elution, the mobile phase A was 0.1% TFA in water; and the mobile phase B was 0.1% TFA in 95% ACN. Proteins were eluted with a gradient of 25–70% B over 100 min at the flow rate of 250 μL/min, followed by a 5-min rinse with 80% B.

The whole protein mixture extracted from rat liver was separated on WAX, WCX and WAX/WCX (1:1, w/w) mixed-bed column with the same HPLC system, respectively. Proteins were separated with the same gradient: 0–15 min of 0% B, 15–25 min of 10% B, 25–35 min of 30% B, 35–45 min of 50% B, 45–55 min of 80% B and 55–65 min of 100% B.

For all HPLC experiments, the analytical columns were maintained at room temperature, and the wavelength for UV detection was set at 214 nm.

3 Results and discussion

3.1 Effect of organic modifier concentration

A mixture of α-casein (0.4 μg/mL, p*I* 4.2–4.7), BSA (1 μg/mL, p*I* 4.7), myoglobin (1 μg/mL, p*I* 7.3), cytochrome *c* (1 μg/mL, p*I* 10.0–10.5) and lysozyme (0.6 μg/mL, p*I* 11.4) was used as the sample. In ion-exchange separation, organic modifiers are often required to suppress the unexpected hydrophobic interaction between proteins and the stationary phase. A commonly used reagent, ACN, was added in the

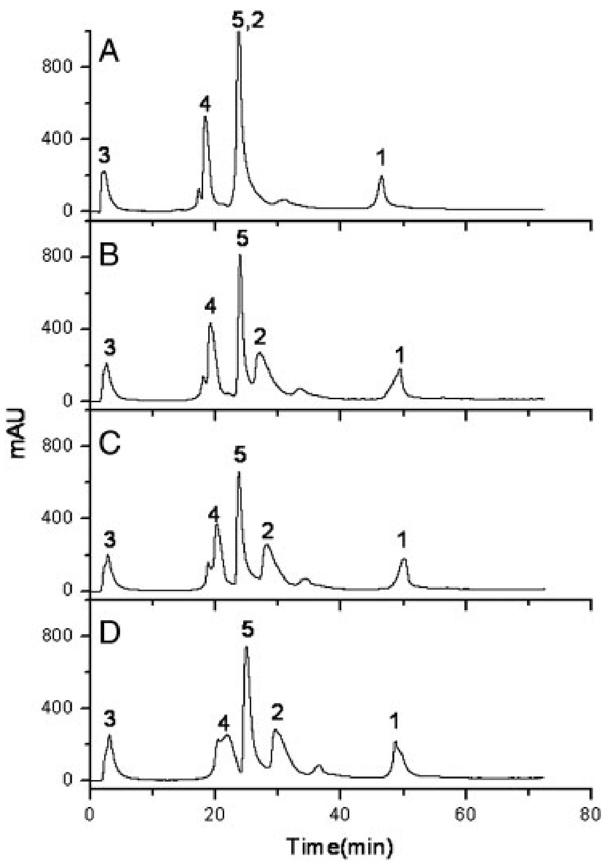

Figure 1. Effect of ACN concentration on protein separation using mixed-bed (WAX/WCX = 1:1, w/w; 0.3 mm id × 100 mm) ion-exchange column. Mobile phase in (A) was A: 10 mM Tris-HCl (pH 8.3); B: A+2 M NaCl (pH 8.3). ACN was added in mobile phases at a concentration of 5% (B), 10% (C) and 20% (D), respectively. Gradient profile: 0–5–75 min, 0–0–70% B. Protein mixture: 1, α-casein; 2, BSA; 3, myoglobin; 4, cytochrome *c* and 5, lysozyme. Injection, 1.5 μL.

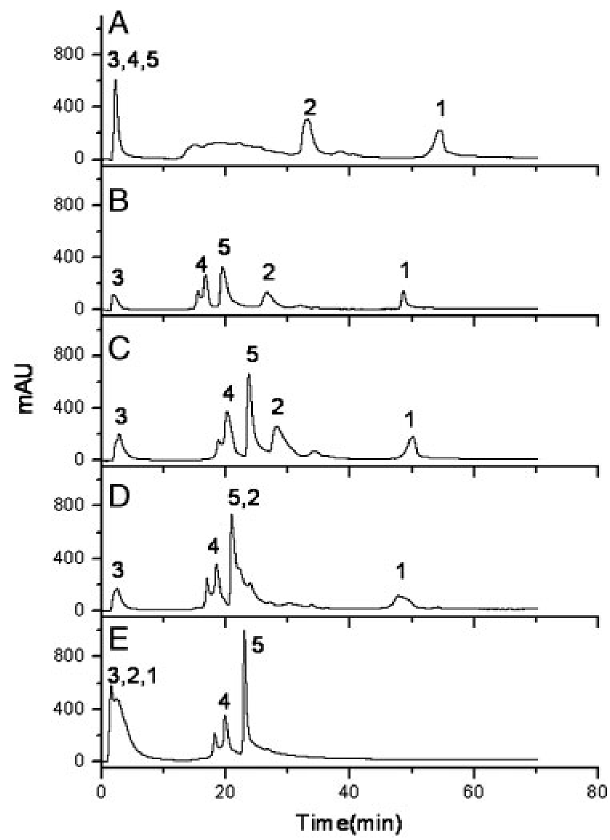

Figure 2. Effect of weak anion/cation packing materials ratio w/w on separation of a five-protein mixture with the ratios of WAX and WCX resin as 1:0 (A), 3:1 (B), 1:1 (C), 1:3 (D) and 0:1 (E). Sample: 1, α-casein; 2, BSA; 3, myoglobin; 4, cytochrome *c* and 5, lysozyme. Injection, 1.5 μL. The chromatographic conditions were the same as in Fig. 1C.

mobile phases as the modifier, and the effect of ACN concentration in mobile phases on protein separation was evaluated, as shown in Fig. 1. It seemed that BSA and lysozyme could not be separated without the addition of ACN, whereas the two proteins were well resolved with the presence of ACN. The maximum resolution of 1.8 was obtained with improved peak shape when 10% ACN was added (Fig. 1C), and the resolutions for other peaks ranged from 1.6 to 7.5. Therefore, 10% ACN was selected as the organic modifier in the following experiments.

3.2 Effect of weak anion/cation packing materials ratio

The ratio of weak anion/cation packing materials is an important factor that affects the retention of proteins. Figure 2 shows the chromatograms of the five-protein mixture separated by columns with various packing ratios of WAX and WCX resins. On WAX or WCX column, either acidic or basic protein peaks were resolved, whereas other proteins were coeluted early. On the contrary, five proteins were resolved well by the mixed-bed columns. This is in accordance with our anticipation that both of the positively and negatively charged proteins could be separated on the stationary phases with blended weak anion/cation-packing materials. Besides, the elution orders of proteins were almost the same (Figs. 2B–D), whereas the profiles were slightly different. This phenomenon might be caused by the different selectivities of stationary phases due to different ratios of packing materials. An optimum weak anion/cation packing ratio of 1:1 was used in the following experiments.

In addition, the relationship between the retention factor and the percentage of WCX ion-exchanger in mixed-bed IEC was investigated, as shown in Fig. 3. For the two positively charged proteins, cytochrome *c* and lysozyme, the retention factor *k* increased roughly as the percentage of WCX was increased from 0 to 1. Oppositely, the retention factor for negatively charged proteins BSA and α-casein decreased with the increase of WCX percentage. This phenomenon was consistent with the retention mechanism of ion-exchange chromatography based on the Coulomb

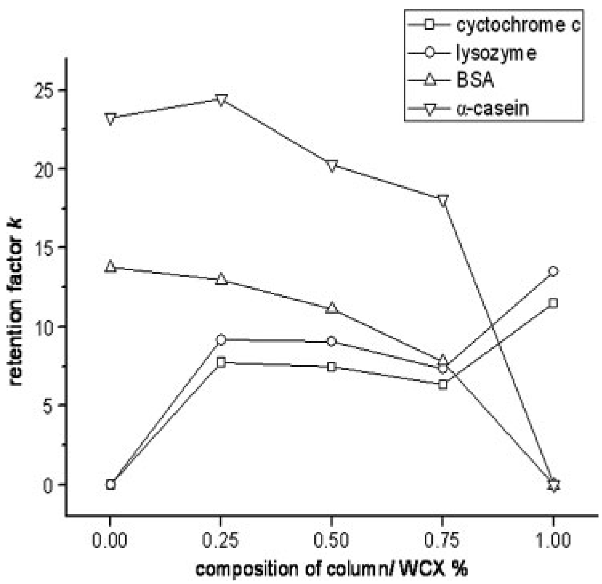

Figure 3. Effect of column composition on retention factor. The conditions were the same as in Fig. 2C.

force. In addition, the nonlinear relationship between the retention factor and the percentage of WCX ion-exchanger in mixed-bed indicated that other interactions might coexist. For example, the charge distribution over the exterior surface of a protein is regarded as heterogeneous and asymmetric, thus the interaction between protein and stationary phase might involve spatial orientation that occurs through a preferred subset of the amino acid sequence [15]. In addition, as suggested by other researchers, molecular weight and aqueous two-phase partitioning coefficients of proteins might also relate to the retention time [16].

3.3 Reproducibility of column packing

The batch-to-batch reproducibility of mixed-bed columns was investigated. The RSD values were calculated in terms of the relative retention time, which were 1.7% for α-casein, 3.3% for BSA, 4.7% for cytochrome *c* and 5.8% for lysozyme ($n = 6$), implying that the packing technique employed herein was reliable and effective.

3.4 Complex sample analysis

Complex protein samples were used to evaluate the applicability of mixed-bed columns (WAX/WCX = 1:1, w/w). One RPLC fraction of proteins extracted from rat middle brain, as shown in Fig. 4A, was collected, lyophilized and resuspended in 50 μL of 10 mM $NaHCO_3$-Na_2CO_3 (pH 8.3) buffer, and further separated on a mixed-bed microcolumn (Fig. 4B). For protein identification, two representative fractions, peaks 1 and 2, were collected and then digested by trypsin. The resulting products were analyzed by a micro-flow RPLC with tandem MS. One protein, II KERATIN KB15 (p*I* 8.0), was identified from peak 1, whereas two proteins, 17 kDa PROTEIN (p*I* 4.1) and CALMODULIN (p*I* 4.1), were identified from peak 2. These results demonstrated that both acidic and basic proteins in the RPLC fraction of complex samples could be further resolved by the mixed-bed IEC microcolumn.

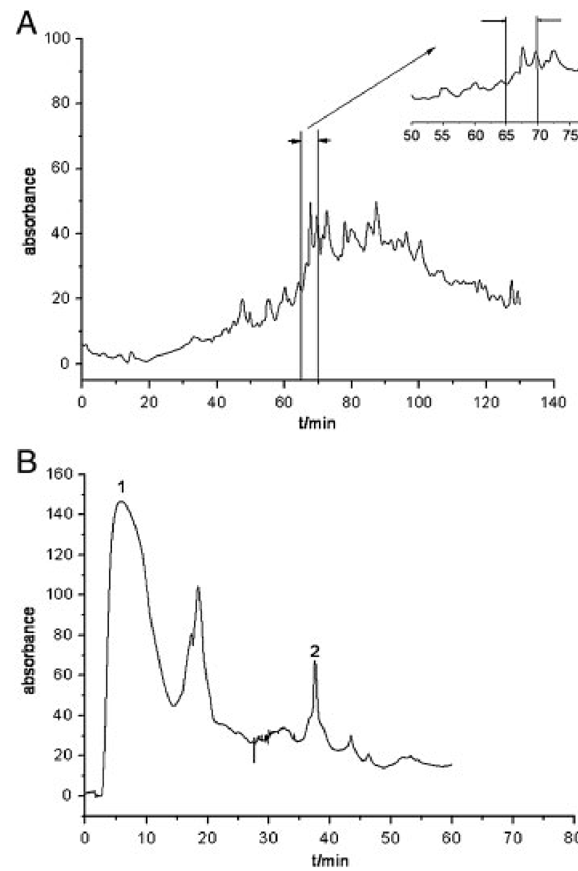

Figure 4. (A) RPLC separation of proteins extracted from rat middle brain. A Hypersil C8 column (4.6 mm id × 250 mm) was used with mobile phase A: H_2O+0.1% TFA and mobile phase B: 95% ACN+0.1% TFA. Gradient profile: 0–100 min, 25–70% B, followed by a 5 min rinse with 80% B; flow rate, 0.25 mL/min and injection, 100 μL. The collected fraction was indicated and amplified on the upper right corner. (B) Separation of one RPLC fraction with the mixed-bed (WAX/WCX = 1:1, w/w; 0.3 mm id × 100 mm) ion-exchange column. Mobile phase A: 10 mM Tris-HCl (pH 8.3)+10% ACN; B: A+2 M NaCl (pH 8.3)+10% ACN. Gradient profile: 0–5–55–60 min, 0–0–50–100% B. Injection, 20 μL.

To demonstrate the improved resolution of the mixed-bed IEC column for more complex samples, proteins extracted from rat liver were separated on WAX, WCX and WAX/WCX mixed-bed column, respectively. As shown in Fig. 5, with 10 μg of proteins injected, more peaks and better peak shape could be obtained by the mixed-bed column. The results demonstrated that proteins with a wide range of p*I* values could be resolved on WAX/WCX mixed-bed column in buffers compatible to tryptic digestion, and

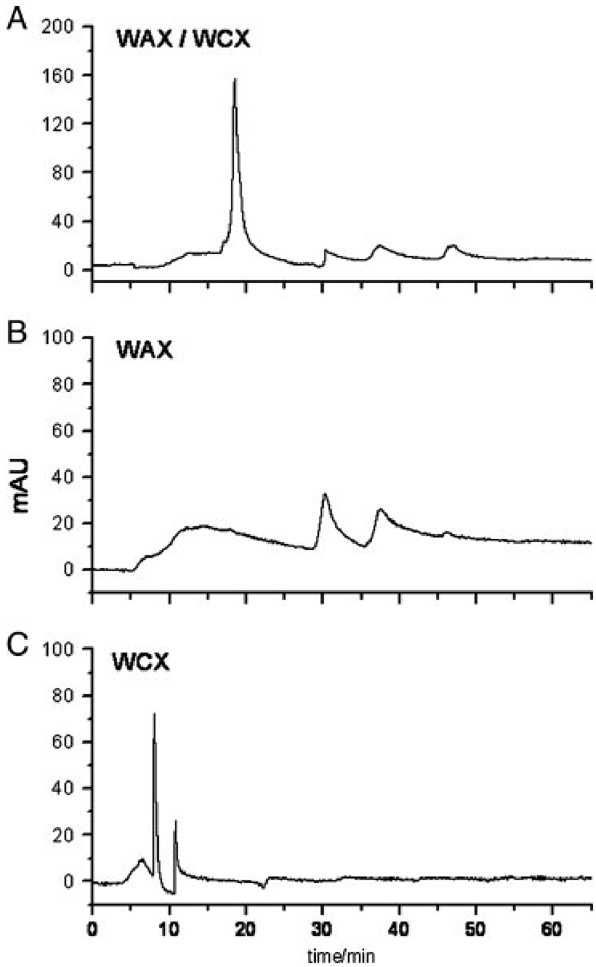

Figure 5. Separation of proteins extracted from rat liver with the mixed-bed (WAX/WCX = 1:1, w/w) ion-exchange column (A), WAX column (B) and WCX column (C). The ion-exchange columns were of the same size of 0.3 mm id $\times$ 100 mm. Mobile phase A: 10 mM Tris-HCl (pH 8.3)+10% ACN; B: A+2 M NaCl (pH 8.3)+10% ACN. Gradient profile: 0–15 min. 0% B, 15–25 min 10% B, 25–35 min 30% B, 35–45 min 50% B, 45–55 min 80% B and 55–65 min 100% B. Injection, 10 μg.

better resolution was yielded than that on either WAX or WCX columns.

4 Concluding remarks

In this study, weak anion/cation mixed-bed microcolumns were developed for protein separation. By the mixed-bed micro-HPLC, both acidic and basic proteins could be resolved in a single run under conditions compatible with tryptic digestion of pH ~8. In addition, the selectivity of the microcolumn could be adjusted by varying the ratio of packed weak anion/cation materials. Unlike RP fractionation, no buffer exchange is required with the mixed-bed IEC. Therefore, this mixed-bed HPLC column is anticipated to be very promising for protein fractionation followed by online tryptic digestion with the advantages of ease-in-operation and less sample loss or contamination. Platforms involving 2-D protein separation (with the mixed-bed column as the second dimension), online digestion, and peptide separation followed by identification with micro-/nano-RPLC-ESI-MS/MS are being constructed in our group, by which the analysis throughput, the detection sensitivity and the accuracy of proteome identification would be largely improved.

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